A. Project Management

A1. Title and Approval Sheet

U.S. Environmental Protection Agency
Office of Research and Development
National Exposure Research Laboratory
Exposure Methods and Measurement Division
Internal Exposure Indicators Branch

QAPP Title:

Ecological Exposure and Biomarkers: Using Advanced Analytical Techniques to Measure Endogenous and Exogenous Biomarkers of Exposure in Support of Modeling Projects

Version 1.0
Effective: May 11, 2018

QA Category: B

ORD National Program: Chemical Safety for Sustainability (CSS)

Project ID: CSS 18.02 Emerging Materials
Project Lead/Organization: Kim Rogers, NERL/EMMD

Project ID: CSS 18.04 Ecological Modeling
Project Lead/Organization: Tom Purucker, NERL/CED

Approvals

Prepared by:
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A2. Table of Contents

A3. Distribution List:

It is the responsibility of the Technical Lead to assure that all project personnel participating or contributing to this research effort receive a copy of the approved QAPP. Copies of this plan and all revisions will be sent to the following individuals in Table 1:

Table 1. Distribution list and contact info.

NAME	ORGANIZATION	TITLE/ROLE	CONTACT INFORMATION
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A4. Project/Task Organization

The work described herein is being conducted by scientific staff in the Exposure Methods and Measurement Division (EMMD) to provide analytical support to collaborators' research efforts in both EMMD and the Computational Exposure Division (CED) (see Table 1). The purpose of this work is to use advanced analytical techniques to measure endogenous and exogenous biomarkers of exposure in support of projects such as those detailed in CSS tasks for Emerging Materials (18.02) and Ecological Modelling (18.04). Overall, data acquired, in conjunction with other biological endpoints, will aid in developing tools for assessing exposures to environmental stressors (e.g. nanoparticles and pesticides) to support ecological risk assessments. These data also support modeling tools for simulating biomarker response to these contaminant exposures in aquatic systems using targeted and non-targeted analytical approaches.

Therefore, the scope of this QAPP includes the laboratory support provided by the Biomarkers Research Team in Athens for biomarker response in ecologically relevant species and tissue residue extraction methods and analytical techniques that

inform parameter estimates used in ecological modeling of amphibians, fish and honeybees.

Matthew Henderson is the overall technical lead of the Biomarkers Research Team and is responsible for overall planning and study design, coordination of sample receipt, in additional to fulfilling the role as a The Biomarkers Research Team member.

Biomarkers Research Team members will be responsible for conducting experiments including laboratory experiments, limited field investigations, sample preparation, instrument operation, data acquisition and analysis, as well as preparation of publications and oral presentations. All Biomarkers Research Team members are responsible for maintaining documentation related to their research activities and implementing the quality assurance/quality control (QA/QC) requirements for sample analysis, data reduction and data analysis as specified in this QAPP and any applicable SOPs, recording and validating results of said experiments in detail, adjusting or calibrating necessary laboratory equipment including analytical instrumentation, and exporting acquired data for use in statistical analysis software packages (e.g. Excel, R, and SIMCA). Furthermore, all members will assume responsibility for verifying results from experiments and instruments that they (or their contracting employees) operate and maintain.

Collaborators are responsible for communicating data needs and analytical requests with the technical lead, coordinating collection and receipt of samples, as applicable, conducting modeling and research efforts outside the scope of this QAPP in accordance with appropriate quality documentation, and assisting with the preparation and review of manuscripts or other scientific and technical products.

Brittany Stuart is the Quality Assurance Manager and is responsible for reviewing and approving the QAPP, performing audits of laboratory activities or data, as appropriate or requested, preparing audit reports, tracking corrective actions, and reviewing and approving scientific and technical products for this effort cleared through EMMD-Athens.

A5. Problem Definition/Background

The Emerging Materials and Ecological Modeling Projects within CSS have multiple, integrated research tasks designed in a trans-disciplinary fashion to harness the expertise across ORD to meet project objectives as described below.

EPA's process for registering and regulating chemical compounds includes a tiered ecological risk assessment (ERA). Within the ERA process, chemicals are first screened using rapid assessment tools that require minimal data and provide conservative estimates of ecological risk. Chemicals determined to present an appreciable risk are subject to higher-level assessments that provide quantitative estimates of ecologically-relevant risk and identify risk mitigation options. For the vast majority of chemicals and species, little or no data exists and refined assessments must rely on modeled estimates of exposure and effects. Historically, exposure assessments rely on environmental monitoring of specific chemicals of emerging concern, however; using advanced analytical techniques to measure endogenous and exogenous biomarkers of exposure will greatly enhance these assessments. Specifically, when unknown chemicals, degradates, or metabolites are responsible for adverse biological effects, efforts described in this QAPP can develop tools and markers of exposure for use in new, improved ecological risk assessments.

Regardless of the Office or Region in which an ERA is performed, they are limited by the amount of reliable data that is available to assess the potential chemical impacts, evaluate risks to environmental resources, and evaluate risk management options. Limited data increases the knowledge-based uncertainties in ERAs (e.g., lack of information on underlying mechanisms, failure to consider multiple stressors, extrapolation beyond the range of observations, etc.) and hinders EPA's ability to evaluate risks of chemical exposure, characterize effects with the necessary spatial and temporal resolution to protect vulnerable populations (e.g., endangered species), and project the performance of risk management options to minimize unacceptable risks. The Agency research need that is addressed through this project is the development and validation of models, through the generation of laboratory data, and approaches that reduce knowledge-based uncertainties using data that are commonly available for ERAs.

The Emerging Materials project area advances the use of functional assays to parameterize environmental fate models of engineered nanoparticles with emphasis on carbon-based nanomaterials (see QAPP entitled: Fate of Nanoparticles in the Environment QAPP, D-EMMD-0031462). Functional assays, which measure intermediate values that integrate nanomaterial properties (e.g. oxidation status) in well-defined reference systems, will be used to generate input biomarker response parameters for exposure models. Specifically, utilizing WASP8, data derived from nanomaterial functional assays and *in vitro* exposures in ecologically relevant species will be integrated with modeled nanomaterial concentrations to develop biomarker response relationships (see QAPP entitled: Transport of Nanoparticles in the Environment: Redesigning the Architecture of the Water Quality Analysis Simulation

Program (WASP) Toxicant Module (TOXI to Advanced Toxicants) to Simulate Nanomaterials in Surface Waters, D-CED-0030090).

The Ecological Modeling project area advances efficient methods to improve risk assessments with limited data availability, as well as more complex approaches that can target data-rich applications. Although the emphasis is on models, these models require parameters that sometimes are not available in the literature for specific compounds or apical endpoints. In these situations, researchers within the ecological modeling project may collect laboratory and/or field data to inform parameter estimation and selection of pesticide models.

Ultimately, research advancing model development and implementation will be anchored to high priority Agency needs using an integrated modeling system that will reduce knowledge-based uncertainties in ERA. Approaches will be designed to improve methods for assessing environmental transport and transformation of new and/or methodologically challenging compounds (e.g., chemicals for which current approaches do not adequately predict or describe risks) and to support efficient assessment for sustainable decisions throughout the Agency. The research will also advance ecological risk assessments for threatened and endangered species, consistent with NAS recommendations (NRC 2013, 'Assessing Risks to Endangered and Threatened Species from Pesticides'). Approaches developed in this research project may be generalized for Agency-wide ERAs, targeted to high priority Program Office needs, or designed for fit-for-purpose application across different Offices and Regions. Research will link ecological outcomes to molecular initiating events through integration with the Adverse Outcome Pathway (AOP) Discovery and Development research and explore approaches for how this linkage enhances risk assessment for chemicals with minimal available data.

A6. Project/Task Descriptions

This task develops and utilizes both targeted and non-targeted, biomarker-based, analytical approaches applicable to exposure scenarios of relevance to EPA's Program Offices and Regions. Endogenous and exogenous biomarker analysis will be conducted using advanced analytical tools such as gas and liquid chromatography coupled with mass spectrometry (e.g. GC/MS; GCxGC/MS, and LC/MS-MS) to elucidate changes in the metabolome of various species and relate these changes to levels of exogenous compounds present. Data derived from biomarker assays, describing the physiological perturbations in living systems, will be used to develop compound specific biomarker-response relationships. Overall, this task uses a combination of analytical tools, advanced statistical analyses, and biochemical

expertise/interpretation to develop tools and markers of exposure for use in environmental and ecological risk assessments.

The primary objective of this effort is to use ecological exposures and biomarker analysis to develop tools for exposure assessment in both laboratory and limited field based studies. Specific goals of this project are described below and each project is briefly defined based on its applicability to the overall task aims.

- **Emerging materials: Developing Exposure Indices of Graphene-based Nanoparticles by Coupling Lipid-Membrane Interactions and *In Vitro* Cellular Response:**
  - Graphene oxide (GO) and graphene-based nanoparticles are increasingly being used in biomedical, environmental, and industrial applications due to their distinct chemical and physical properties. Their widespread use and application can potentially result in mass release and exposure of GO to the aquatic environment. Understanding the environmental transformation and fate of GO is necessary to accurately assess its ecological impact. Studies have demonstrated that GO is cytotoxic in numerous species but few have focused on investigating GO toxicity under environmental conditions. The objective of these studies are to investigate the exposure potential of GO and its transformation products in ecologically relevant species. *In vitro* biomarker-based approaches are utilized to identify pathways involved in GO exposure and toxicity. These studies, with identification of GO transformation products (e.g. PAHs), will aid in assessing the ecoexposure potential and ecotoxicity associated with this class of materials.
  - Environmental studies have indicated that GO is stable in the water column even at relatively high ionic strengths and that GO may be phototransformed to yield both reduced GO products (rGO) as well as oxygenated PAHs. Also as part of this study, simulated water body GO concentrations will be assessed for their potential ecological effects using model membrane systems: GO interactions with phospholipid supported bilayers as well as bilayers derived from fathead minnow (Pimephales promelas) cell membrane extracts.

- **Emerging materials: Assessing the Exposure and Toxicological Implications of Environmental Transformations of Graphene Oxide Using *In Vitro* Methods:**
  - Biological responses (transcriptomics, proteomics and metabolomics) often represent immediate or early responses within the organism and may be associated with signaling networks that are linked to adverse outcomes at higher levels of biological organization. For fish, gene expression, metabolomics, and protein biomarkers are being developed that target carbon based nanomaterial

exposures. These biomarkers are used to evaluate the accuracy of predicted internal dose measurements, which are determined using a series of environmental fate, uptake and biotransformation models. Additionally, biomarker responses will be aligned with adverse outcome pathways (AOPs) and effects models being developed within the AOP project and in other tasks within this project. Overall, the objective is to develop cell-based biomarker tools for high throughput screening of carbon nanomaterials for rapid, cost effective exposure assessments.

- **Ecological Modeling: Estimating amphibian pesticide body burden following exposure Investigating Pesticide Uptake and Metabolism Following Dermal Exposure in Terrestrial Phase Amphibians**
  - For terrestrial amphibians, accumulation of pesticides through dermal contact with contaminated soils is a primary route of exposure in agricultural landscapes and may be contributing to widespread amphibian declines. Dermal exposure presents a potentially significant but understudied route for pesticide uptake in terrestrial amphibians. These studies will measure dermal uptake of pesticides of varying hydrophobicity in post-metamorphic frogs. All amphibians will be measured for body burdens at the end of the exposure (as well as pesticide degradates) using targeted biomarker profiling. Dermal uptake data can be used to improve risk estimates of pesticide exposure among amphibians as non-target organisms.
  - Machine learning techniques and other advanced chemometric techniques will be employed to identify biomarkers in American toads, Grey tree frogs and Leopard frogs following exposure to high priority pesticides. Lab experiments will be performed to expose tadpoles to defined dose levels. Metabolomic responses will be quantified using GC/MS. Classification methods will be used to identify biomarkers in each species. Conservation of biomarkers between the amphibian species will then be assessed for consistency of response between these amphibian species. This task will create an enhanced predictive capacity for using inherent chemical properties to parameterize key indicators of environmental fate, biological dose, and ecological effects to support Agency evaluation of a wide range of compounds

- **Ecological Modeling: Fish-Amphibian Translation of Biomarkers to Inform Toxicity Extrapolation (experimental data and manuscript)**
  - This project will coordinate multiple ‘omic responses across fish and amphibian species to provide context for when it may be appropriate to extrapolate quantitative toxicological endpoints from fish to amphibians. Conservation of biomarkers between taxa will then be assessed for consistency of response

along with the consistency of toxicity extrapolation information between these groups (e.g., using Web-ICE). Tissue residue extracts will be split and used by Athens for assessing metabolomic response, Gulf Breeze for proteomic response, and Cincinnati for genomic/transcriptomic analyses. Associated QAPPs include GED-BRPB-JA-2015-01-001; Toxicity Translators: Larval Fish and Tadpole Exposures, and E-ISTD-0031419-QP-1-0; Chemical signatures of neurodevelopmental disorders. New QAPPs will reference the current QAPP and/or will be added as an amendment to the current QAPP as necessary. Impacted biochemical pathway/network identification will proceed within each of these domains and consistency in response will be assessed. This task will develop physiologically-relevant markers of exposure using a combination of analytical tools and biomarker platforms to develop/refine AOPs for chemicals of emerging concern to the EPA.

- **Ecological Modeling: Parameter Estimation and Sensitivity Analysis for Honeybee Colony Simulation Modeling**
  - This project consists of ORD contributions to a collaboration between OPP/EFED (Kris Garber and Andrew Kanarek), USDA (Gloria DeGrandi-Hoffman and Bob Curry), Region 5 personnel and a Regional Applied Research Effort (RARE) with Ohio State (Reed Johnson) to extend and parameterize an existing USDA honeybee colony simulation model, VarroaPop, for estimation of neonicotinoid exposure and effects (see QAPP entitled: Development of Honey Bee Colony Simulation Model for use in Ecological Risk Assessments of Pesticides, D-CED-0031022). The colony simulation model is part of a proposed framework for assessing risks of pesticides to bees (EPA/HC/CalDep White Paper 2012). The proposed framework is similar to risk assessments used by EFED for other taxa, relying on a tiered approach that starts with laboratory-based studies conducted at the individual level and increases in complexity to semi-field and field testing when risks are not precluded at the lower tiers. The model is being extended to integrate pesticide applications (foliar spray, soil, and seed) to the simulations with honeybees exposed to active ingredient by physical contact (i.e. foraging) or ingestion.
  - As part of this project, field samples and controlled lab study data are analyzed to assist in model parameterization. In controlled lab experiments, doses of pesticides will be given at predetermined levels to allow for measurements in different hive media and different bee life history stages. Parameters from the field and nuc studies that can be informed by data collection include temporal and taxonomic variability of neonicotinoid loads on bee-collected pollen, larval and nurse bee survival under controlled dosage levels, neonicotinoid concentrations and related degradates in hive elements, and estimates of

foraging bee mortality. These measurements will be mapped to input variables required by VarroaPop to inform both their point estimates and estimate probability distributions.

- **Miscellaneous Projects and QAPP Amendments**
  - Numerous legacy and 'new' projects can also be covered under this QAPP with necessary amendments. Non-EPA initiated, on-going projects such as pharmaceutical analysis in oysters, rainwater analysis for agricultural use pesticides, antimicrobial analysis in streams and sediments, biomarker analysis of neuronal cells exposed to neuroactive compounds, etc. are all required to follow the policies and procedures outlined in this QAPP. The current QAPP will be amended as new projects falling under the research objectives of and deemed adequate by the Biomarkers Research Team are added.

A7. Quality Objectives and Criteria for Measurement Data

For non-targeted biomarker studies, data from sample extracts will be collected using LC/MS or GC/MS operated in full scan mode (m/z 50-650). The end results of these analyses are numerous measurements of endogenous metabolite concentrations (reflected by peak area or height) that reflect the biochemical state of the cell, tissue or biofluid. By comparing measurements of these metabolites between exposed and non-exposed organisms, the impacts of stressors on the biochemistry and physiology of the exposed organisms can be determined. As a result, it is critical that only relevant metabolite changes are included in the final results that are reported. For this reason, the Biomarkers Research Team employs appropriate statistical tests to determine the significance of metabolite changes relative to controls after determining that all quality criteria are met (see Section B5 Quality Control).

For targeted biomarker analysis, the Data Quality Objectives (DQOs) are to apply analytical methods (e.g. LC/MS-MS) which can achieve a target method limit of detection of <1 ng/g (<1 ppb) for pesticides and other chemicals of concern, and <10 ng/g (<10 ppb) for metabolites balanced against method cost, efficiency and reliability. The Biomarkers Research Team recognize that the achievement of quality data depends upon an effective and consistent QA program. The implementation of the QA program will be achieved through a team effort by the entire laboratory group and QA staff.

The general considerations and objectives of the Biomarker Research Team overall QA program are as follows and each are detailed in subsequent sections of the QAPP:

- Sample integrity should be preserved by following documented sample handling procedures relating to sample preservation, holding times, storage, labeling and record keeping associated with all samples, where applicable.
- Analytical instrumentation should be maintained in proper working order following the manufacturer's guidelines to meet the targeted level of detection.
- Instrument performance, calibration, and maintenance should be verified and documented in the research notebook or instrument logbook.
- The analytical bias (in terms of % recovery) and precision (in terms of relative standard deviation (RSD)) of quality control samples as part of analytical methods should be recorded and maintained on a continuing basis.

The following sections describe the standard workflow that is used for assessing multivariate and univariate statistical significance and constitutes the principal method that will be used as measures of quality for reporting (see QAPP entitled: Metabolomics: The Use of Advanced Analytical Tools to Identify Markers of Exposure and Link Exposures with Whole Organism Outcomes, D-EMMD-0031335). Results that are deemed statistically significant will be reported and identified as putative metabolite identification for biomarker discovery and analysis. Results that are not significantly will not be reported as described in D-EMMD-0031335.

A7.1 Multivariate Statistical Approaches

Multivariate statistical analysis will be performed by importing Excel spreadsheets of binned chromatograms into SIMCA-P+ (Umetrics Inc., Umea, Sweden) or other appropriate multivariate software (e.g. Metaboanalyst; www.metaboanalyst.ca). Preliminary principal component analysis (PCA) will be conducted for the entire dataset using mean-centered and Pareto-scaled bins. The relative impact of a given chemical exposure will be assessed by comparing score values for different treatment classes within a given PCA model. To make these comparisons, the score values for a given component (e.g., PC1 scores in a PCA model) will be tested for normality using the Kolmogorov–Smirnov test. If found normal, then a one-way ANOVA test, with Tukey's post hoc comparison, will be conducted. This allows the determination of significantly different means (95% confidence) of score values for different treatment classes. All statistical tests on score values will be performed with Minitab 16 (Minitab, Inc., State College, PA, USA). Statistical significance will be ascribed to score values with a p-value < 0.05.

A7.2 Univariate Statistical Approaches

Using the Excel spreadsheet of binned chromatograms, univariate statistical tests will also be conducted to determine the significance of metabolite changes resulting from the exposures. For GC/MS and LC/MS data, alignment programs can be run on the data set prior to univariate statistical approaches. The developers recommended, instrument specific parameters will be used as necessary (Niu et al., 2014).

To generate a filtered chromatogram of significant peaks, first an "average class spectrum" will be calculated by averaging the binned chromatograms across all class members, where class is defined by exposure level (including controls) and duration. Next, a difference spectrum will be generated by subtracting the averaged bins of the relevant control class from those of each exposed class. Then, a Student's t-test will be conducted on each bin using a p-value < 0.05. To greatly reduce the rate of false positives, the following corrections were made to accommodate multiple testing in determining p-values (Collette et al., 2010). Any single isolated bin that passed the t-test (without an adjacent bin also passing) will be replaced with a zero (i.e., it is rejected), because legitimate metabolite peaks span more than one bin at this bin size. To further reduce false positives, any occurrences of two (and only two) adjacent bins with opposite arithmetic sign will be replaced with zeros, because this outcome is incompatible with NMR peak shapes. The result is a "t-test filtered difference chromatogram" for each exposed class. Positive peaks in these difference chromatograms correspond to metabolites that increase (with statistical significance) upon treatment, whereas negative peaks represent metabolites that decrease.

A8. Special Training Needs/Certification

A8.1 Basic Laboratory Training

This document assumes laboratory personnel will have a thorough working knowledge of basic laboratory skills, reagents, and instrumentation. The technical lead will ensure that personnel joining the Biomarkers Research team have fulfilled the following basic training requirements prior to pursuing additional technical training as described in A8.2:

- Initial and annual refresher training on proper laboratory or field safety and health procedures in place as specified in the Health and Safety Plan.
- Orientation training on ORD and NERL quality system requirements related to recordkeeping and laboratory practices to include, but not limited to:
  - ORD Policies and Procedures Manual Section 13.02 Scientific Recordkeeping: Paper

  - ORD Policies and Procedures Manual Section 13.04 Quality Assurance/Quality Control Practices for ORD Laboratory and Field-Based Research
- Designated personnel working on this project have received, at a minimum, familiarity training on the operation of the analytical instruments

Records of this training are maintained by individual researchers and documented in their research notebooks or are maintained by the onsite Safety, Health and Environmental Management or QA office.

A8.2 Technical Training

Training of researchers on new activities, and of new researchers, will be performed by:

1) independent review of quality documentation (e.g. QAPP), standard operating procedures and manufacturer instructions manuals; (Manufacturer instruction manuals and schematic diagrams are also maintained in the laboratory as are official training documents for each instrument.)
2) remote training and/or short courses;
3) on-site short courses provided by instrument vendors; or
4) experienced research team members by demonstration followed by close supervision until the activity is mastered.

Mastery of an activity will be determined by direct observation and scrutiny of the learner's analytical results for known standards. Competency of trainees will be documented in the Research Notebook and data acquisition for research will not occur prior to analyst training. Competency will be determined by a combination of analyzing QC samples of commercially available tissues (i.e. chicken liver) and/or conducting percent recovery experiments for targeted chemical analysis. Following competency training, certification of the trainees will be documented by the PI, or appropriate surrogate, signing their research notebook.

A designated user who has been properly trained will be responsible for all sample analyses and instrument maintenance to insure consistency of operation (in accordance with manufacturer recommendations). As it stands, Matthew Henderson, assumes responsibility for training all current and future members of the Biomarkers Research Team on the Agilent 5795 GC/MS, Agilent 6020 LC/MS, Varian 1200L LC/MS-MS, Thermo Quantum AM LC/MS-MS, Waters Autospec Mass Spectrometer (GC/MS), LECO Pegasus 4D GCxGC/ToF-MS, and any other routine instruments used in research efforts under this QAPP.

A9. Documentation and Records

Details regarding all aspects of experiments will be recorded in the research notebooks as required by ORD Policies and Procedures Manual (PPM) Section 13.02: Scientific Recordkeeping: Paper, unless otherwise noted. Records for all laboratory generated data, all pertinent procedures, raw data acquisition, and calculations, etc. are maintained in the research notebook according to the aforementioned PPM. Data reduction and summation records will be maintained electronically on computer disks and flash drives.. Hard copies of the instrument tune and calibration files will be kept in instrument specific notebooks or electronically in a designated folder on the controlling computers desktop. GC/MS, GCxGC/ToF-MS, LC/MS, and LC/MS-MS data will be backed-up either on the LAN or flash drives, or other appropriate media. When available, all instrument controlling computers are equipped with a secondary hard drive to back up raw, instrument-specific data files will be conducted semi-annually or at the conclusion of all data acquisition of individual projects. All data collected by computer will be given a unique name with the date encoded therein (e.g. YYMMDD###).

When data reduction is not computerized, the calculation will be recorded in research notebooks. All reports and documentation required, including calibration records, and QC results will be labeled with the corresponding sample set number and date. All information associated with an experiment is stored in MS Windows directories that are referenced to the proper experiment or study in the research notebook. Generally, data stored in digital format will be backed-up over the network. Furthermore, applicable SOPs are referred to in the research notebook, as well as a listing of applicable QAPPs. SOPs are maintained by the EMMD QA Manager, as well as by the PI and are located on the respective PI's assigned work computer or in the research notebook. Relevant results will be published in refereed journals or EPA reports.

Research activities should be documented in research notebooks as specified in organizational quality system procedures.

- Refrigerator/freezer logs are monitored and recorded weekly on a logsheet secured to the front of the unit.
- Upon completion of the project, and while the project is ongoing, appropriate project files including all chromatographic data should be copied to electronic media for long-term storage and included with the project files.
- Upon completion of the project, all project records, including electronic media, will be stored as specified in the applicable Records Retention Schedule.
- The QAPP will be distributed, and redistributed as changes and updates are made, to all individuals listed on the distribution list (section A3).
- Records of communication will be kept in the project files.

B. Data Generation and Acquisition

B1. General Experimental Design

Dosing studies (e.g. amphibian exposures and cell culture) and sample collection (e.g. surface water samples) will be conducted by trained and certified EMMD personnel according to published standard operating procedures (SOPs). All animal studies are animal use protocol (AUP) approved and procedures are described in relevant SOPs (see section B2). Environmental sampling methodologies are also described in relevant protocols and SOPs (see section B2). Following collection, samples will be transported back to the ORD laboratory in Athens for timely processing, and LC/MS-MS or GC/MS analysis.

Note that most (>80 %) of samples and exposure studies are conducted at other NHEERL or NERL laboratories. These laboratories will be responsible for maintaining individual QAPP and associated SOPs according to their own QA requirements. Copies of these associated QAPPS will be electronically requested by the PI, cited in an amendment to the current QAPP, as necessary, as stored electronically by the PI.

B2. Sampling Methods

B2.1 Animal/Tissue Samples

Dosing of animal and tissue samples received under collaborative efforts are not applicable to this QAPP; therefore, the technical lead will assume all samples have been collected according to the affiliations required protocols and SOPs.

All rearing, housing and animal studies are done in accordance with approved animal use and care protocols (e.g. AUP # for all amphibian related biomarker studies). SOPs associated with EMMD and CED sample collections are listed in Table 2. Tissues will be extracted following the SOP titled Processing Fathead Minnow Tissues in 96-well Plates for metabolomics (D-EMMD-IEIB-SOP-892), reconstituted for LC/MS analysis or derivatized for GC/MS analysis per each SOP as required (Table 3).

B2.2 Cell Culture Samples

Cell culture exposure experiments will be performed in the cell culture laboratory under the direction of PIs. Cell culturing, dosing and sample collection will be conducted by trained personnel according to published SOPs presented in Table 2.

Culture medium of cells will be changed at least twice weekly. For cell exposure tests, cells will be harvested and divided for different exposure classes and times. After

the exposure tests are completed, the cells will be quenched with 80% methanol in an approved bio safety cabinet and kept in cold racks for extraction (Teng et al 2009). Quenched cells will be extracted according to D-EMMD-IEIB-SOP-941. Media from cell culture experiments will be quenched with acetone (10%) and stored until derivatized for GC/MS analysis using the D-EMMD-IEIB-SOP-1922 in Table 3.

B2.3 Environmental Samples

Environmental samples will be collected based on approved SOPs listed in Table 2 or collaborator's associated SOPs outside the scope of this QAPP.

Table 2: List of SOPs associated with Sample Methods included in the current QAPP.

SOP Number	Title	Author
D-EMMD-IEIB-SOP-941-1	Chemical Exposure Using Cell Cultures	Teng
D-EMMD-IEIB-SOP-1954-0	Amphibian Rearing and Husbandry	Purucker
D-EMMD-IEIB-SOP-1955-0	Stem Flow and Pond Water Sampling	Purucker
D-EMMD-IEIB-SOP-1956-0	Field Amphibian Sampling	Purucker
D-EMMD-IEIB-SOP-1957-0	Amphibian Exposure Studies	Purucker

B3. Sample Handling and Custody

Samples received by the Principal Investigators (PI) will be shipped overnight on dry ice, or other appropriate refrigerant as deemed necessary to ensure sample integrity, and inspected upon receipt for integrity and stored at a nominal temperature of -80°C. Similarly, all cell culture samples generated at EMMD Athens, GA will be stored at -80ºC until the time of analysis. A sample log will be maintained containing information about each sample received such as date of arrival, sample physical state (whether frozen or not), name and initials of person receiving samples, etc. Remaining samples after analysis will be stored frozen at - 80°C until the completion of the project. Most samples will be processed and analyzed within three months of receipt, however, extended periods may be warranted without loss of data integrity due to low temperature storage at <-80 °C.

Chain of Custody forms for all samples received from collaborators will be bound and maintained in the PIs' laboratories. Each sample will be given a unique number so that proper identification can be maintained throughout the laboratory study. For non-EMMD generated samples, the sending institution will provide their chain of custody (CoC) from when shipping/delivering samples to EPA. The EPA researcher will sign the CoC and keep a copy for record. Chain of custody forms will be shipped with samples and include information such as collected by name/date, shipped by name/date, and

received by name/data plus any additional sampling information necessary to document sampling location, experimental design, and any relevant sample comments.

Laboratory experiments will be performed in laboratory space under the direction of the PIs. Sample preparation steps and analysis also will be documented so that a sample's history in the laboratory can be readily examined. An instrument log documenting performance and maintenance will be kept for the instruments used for sample preparation.

B3.1 Handling and Preparation of Biomarker Samples for GC/MS Analysis

Endogenous metabolites are extracted and analyzed following the bi-phasic procedure described in Viant (2007). Briefly, tissues are extracted with a mixture of methanol and chloroform using 2 mm beads in a tissue homogenizer to separate metabolites into a polar and non-polar fraction. Individual fractions are placed into 2 mL vials and evaporated to dryness overnight on a speedvac. Samples are derivatized with 100 µL of methoxyamine hydrochloride in pyridine (20 mg/mL; 2.5 h, 60 °C) and then with 100 µL of BSTFA containing 10% TCMS (1.5 h, 60 °C) for a total of 2.5 h. After derivatization, all polar samples are analyzed via gas chromatography coupled with mass spectrometry as in attached SOPs.

B3.2 Handling and Preparation of Biomarker Samples for LC/MS (LC/MS-MS) Analysis

Endogenous metabolites are extracted per SOPs listed in Table 3 (i.e. D-EMMD-IEIB-SOP-892-1). After samples are evaporated to dryness overnight on a speedvac, samples will be reconstituted in 10% acetonitrile for LC/MS or LC/MS-MS analysis.

B3.3 Handling and Preparation of Environmental Samples for GC-MS Analysis

Environmental samples will be processed using established SOPs in Table 3 depending on target analyte and matrix studied. For example, the processing method of surface water samples is described in D-EMMD-IEIB-SOP-1920. Briefly, all water samples will be filtered through a 0.45 µm GF/F filter paper using a filtration apparatus. The entire sample will be passed through a pre-conditioned C18 solid phase extraction (SPE) cartridge (Oasis HLB 6cc, 500mg) at a rate of 10 mL min-1 that is attached to a vacuum manifold. The SPE will be dried under vacuum for 45 minutes and then analytes eluted sequentially with 6 mL each of methanol and dichloromethane into a glass disposable cell culture tube. The sample will be gently evaporated under nitrogen gas and then reconstituted in 1 mL of ethyl acetate and transferred to a 2 mL vial prior to GC/MS analysis.

B3.4 Handling and Preparation of Environmental Samples for LC/MS (LC/MS-MS) Analysis

Exogenous metabolites for LC/MS-MS analysis will be processed and handled based on approved SOPs listed in Table 3. (i.e. D-EMMD-IEIB-SOP-1920-0, D-EMMD-IEIB-SOP-1959-0 or D-EMMD-IEIB-SOP-1946-0, as necessary). Following gentle evaporation to dryness with nitrogen gas, samples will be reconstituted in 1 mL 10% acetonitrile for LC/MS or LC/MS-MS analysis.

B3.5 Tissue and Cell Extractions Procedures

Frozen (<-70^{o}C) intact liver tissue samples (~20 mg) will be extracted using SOP titled "Processing Fathead Minnow Tissues using two 96-Well Plates (D-EMMD-IEIB-SOP-892). Extracts are dissolved in 10% aqueous acetonitrile prior to submitting for LC/MS-MS analysis or derivatized following derivatization SOPs listed in Table 3 for GC/MS analysis.

All cell culture samples will be extracted using a SOP titled Chemical Exposures using Cell Cultures (D-EMMD-IEIB-SOP-941). Briefly, dry polar cell extracts (either test or control) will be thawed at room temperature for 5 minutes before being reconstituted for LC/MS analysis or derivatized for GC/MS analysis.

Table 3: List of SOPs associated with Preparation of Biomarker samples for GC/MS or LC/MS^{n} included in the current QAPP.

SOP Number	Title	Author
D-EMMD-IEIB-SOP-892-1	Processing Fathead Minnow Tissues Using two 96-well Plates	Mosley
D-EMMD-IEIB-SOP-1920-0	Extraction of Environmental Contaminants from Water Samples Using Oasis Hydrophilic-Lipophilic Balance (HLB) Solid Phase Extraction Cartridges	Henderson
D-EMMD-IEIB-SOP-1946-0	Extraction of Pesticides from Amphibians	Henderson
D-EMMD-IEIB-SOP-1947-0	Extraction of Pesticides from Soils	Henderson
D-EMMD-IEIB-SOP-1958-0	Extraction of Neonicotinoids from Pollen and Beebread	Purucker
D-EMMD-IEIB-SOP-1959-0	Extraction of Neonicotinoids from Honey Bees	Purucker
D-EMMD-IEIB-SOP-1960	Extraction of Neonicotinoids from Honey	Purucker
D-EMMD-IEIB-SOP-1923-0	Derivatization of Metabolomic Extracts for Analysis by Gas Chromatography Coupled with Mass Spectrometry (GC/MS)	Henderson
D-EMMD-IEIB-SOP-1922-0	Derivatization of Cell Culture Media for Analysis by Gas Chromatography Coupled with Mass Spectrometry (GC/MS)	Henderson

B4. Analytical Methods

Major instrumentation required for biomarker analysis include but is not limited to GC/MS and LC/MS. General preparation of tissue samples for endogenous biomarker analysis includes extraction and derivatization prior to instrumental analysis as described in Section B3 and detailed by the SOPs listed in Table 3. The analytical methods developed for the extraction and cleanup of tissue residues (amphibian samples, bee and bee product samples, fish samples, associated soil and water samples) include using solid-phase extraction or liquid-liquid extraction followed by liquid chromatography/ tandem mass spectrometry (LC-MS/MS) will be the primary procedures used. The analytical SOPs used in this project are summarized in Table 4. SOPs will be generated and added as required by new projects approved by the Biomarkers Research Team.

B4.1 LC/MS

LC/MS analyses will be conducted on either an Agilent 6120 LC/MS or a Thermo LC/Quantum AM mass spectrometer in electrospray ionization (ESI) mode. The column chemistry and mobile phase gradient will be adjusted based on the analyte(s) under investigation. All methods are available on the instrument computer and are named according to the tissue/biofluid being studied and class of analyte(s) being studied. See SOP titled “Metabolomics: LC-MS Analysis of Tissue/Biofluid Extracts” for further details. Specific instrument parameters that are analyte-specific are recorded in the instrument log book and reserach notebook of PI (or approved user).

B4.2 GC/MS

GC/MS analysis will be conducted on either an Agilent, Leco or Water gas chromatography coupled to a mass spectrometer. All GC-MS spectrometers will be tuned and calibrated using perfluorotributylamine (PFTBA), according to the manufacturer’s instructions, before each sample set. Sample extracts will be analyzed under full-scan (*m/z* 50-650), electron ionization-MS conditions. Specific instrument parameters that are analyte-specific are recorded in the instrument log book and research notebook of PI (or approved user).

Table 4: List of SOPs associated with Analytical Methods included in the current QAPP

SOP Number	Title	Author
D-EMMD-IEIB-SOP-1948-0	Using Gas Chromatography Coupled to Mass Spectrometry (GC/MS) for the analysis of Metabolomic Samples	Henderson
D-EMMD-IEIB-SOP-1949-0	Liquid Chromatography-Tandem Mass Spectrometry for the Analysis of Endogenous Biomarkers	Henderson
D-EMMD-IEIB-SOP-1950-0	LC/MS-MS Analysis of Environmental Contaminants	Henderson
D-EMMD-IEIB-SOP-1951-0	Analysis of Neonicotinoids using LC/MS-MS	Henderson
D-EMMD-IEIB-SOP-1952-0	Using Two-dimensional Gas Chromatography Coupled to Time of Flight Mass Spectrometry (GCxGC-ToF/MS) to Analyze Pesticides	Henderson
D-EMMD-IEIB-SOP-943-1	Metabolomics: LC-MS Analysis of Tissue/Biofluid Extracts	Ekman

B5. Quality Control Requirements

All Biomarker Research Team members are responsible to ensure the following are met before reporting results. Ultimately, each team member should use his/her best judgment and experience, as well as the manufacturer's guidelines, to properly maintain the instruments. Preventative maintenance and repairs shall be written in the instrument logbook.

For targeted biomarker analysis requiring the use of stock solutions and standards, stock solutions will be retained in glass or plastic containers and labeled with the date, name of the preparer, and the concentration of the solution.

B5.1 GC/MS Data Collection and Analysis

The Agilent mass spectrometer (7890 GC with 5973 MSD) will be tuned and calibrated using perfluorotributylamine (PFTBA), according to the manufacturer's instructions, before each sample set (no more than 50 per run). In addition to tuning and calibrating the GC/mass spectrometer daily, a QC mix of amino acids (a mixture of the amino acids aspartate and asparagine prepared in 50% aqueous methanol and derivatized following the same protocol as for the samples) will be run after every 50 injections to ensure good chromatography and sensitivity of the GC/mass spectrometer. If the chromatography is poor (reduced retention time (> 0.2 minutes), poor peak shape, co-eluting compounds, or baseline drift or the sensitivity is sufficiently low compared to the previous analysis of the QC mixture (>10% reduction in peak area and/or peak area ratio differences of >10%), remedial actions are taken, such as trimming the column,

replacing the injection port liner, o-ring, and septum. If these actions do not sufficiently resolve the LC and MS issue(s), then a new GC column is installed and/or the EI source is cleaned.

GC/MS conditions will be as follows. The column will be conditioned and the system equilibrated prior to each set of samples being analyzed by injecting six pooled QC samples (the data from these QC samples are not analyzed; these QC samples are used solely to condition the column and equilibrate the system). Next, a method blank is run, followed by a pooled QC sample. The study samples are then analyzed in random order to prevent any batch effects. A method blank and a pooled QC sample are run at the beginning of the sequence, at the end of the sequence, and after every 10 study samples.

Internal standards and external standards (dependent on project use) are carefully examined prior to data processing of the study samples to assess the derivatization efficiency and to assess overall instrument performance. Acceptance criteria are also variable dependent on the goals of each individual project and will be documented in the PIs laboratory notebook. Generally accepted acceptance criteria are >75% recovery of internal standards, >15% RSD for QAQC samples, and an r2 >0.9 for linear regression of external standards. Samples and/or datasets not meeting these criteria will be flagged for re-analysis or data un-reportable.

B5.2 LC/MS Data Collection and Analysis

The Thermo LC/Quantum AM mass spectrometer will be tuned according to the physiochemical properties of the analyte(s) under investigation and calibrated every 24 hours, prior to each discrete data set, or weekly if instrument is idle using commercially available tuning compounds (e.g. PPG). Passing a tune, based on the manufacturer's recommended parameters (e.g. 'Signal Stability > GOOD), is required before operation of the instrument can commence. Furthermore, if the chromatography is poor (reduced retention, poor peak shape, co-eluting compounds, or baseline drift), the mass error is greater than 2 ppm, or the sensitivity is sufficiently low, remedial actions are taken, such as replacing the guard column, flushing the LC column with solvents, back flushing the LC column, changing the LC tubing, cleaning the injection port rotor, stator, and injection valve sleeve, and/or cleaning the mass spectrometer sweep cone, ion transfer tube, and ESI needle. If these actions do not sufficiently resolve the LC and MS issue(s), then a new LC column is installed and/or the entire electrospray source and RF lens stack will be cleaned.

The LC column will be conditioned according to manufacturer recommendation and the system equilibrated prior to each batch of samples being analyzed by injecting 10 pooled QC samples (the data from these QC samples are analyzed; these QC samples are used solely to condition the column and equilibrate the system). Next, a method blank is run, followed by a pooled QC sample. The study samples are then analyzed in random order to prevent any batch effects. A method blank and a pooled QC sample are run at the beginning of the sequence, at the end of the sequence, and after every 10 study samples. See Section B5.4 for further details on QC samples for targeted biomarker analysis.

B5.3 Metabolite Identification

Peak lists will be created from raw GC/MS data files. All peaks corresponding to column bleed, derivatizing reagent by-products, internal standards, external standards, and peaks present in the method blanks are removed from the final peak list. All entries in the peak list that correspond to the same metabolite are summed and listed as a single metabolite (i.e., the abundances of the various isomers of glucose detected are summed and listed as a single entry for glucose). The data will then be normalized to the unit sum of 1 (or TIC).

To determine the identities of the metabolites measured in each sample a library search for each metabolite detected will be conducted using our custom library. This in-house library contains 1175 mass spectral tags, 583 of which are non-redundant. There is a fair amount of redundancy because many metabolites have differing degrees of derivatization and/or exist in various isomeric forms. For example, there is more than one entry for glucose in the library because there is a separate entry for each isomer. This is because each isomer, while having identical mass spectra, will have different retention time/retention indexes. Our library accounts for these scenarios and can identify the specific isomer. Each mass spectral tag in our library contains the metabolite name, mass spectrum, retention index based on 8 evenly eluting n-alkanes, and the corresponding quantifier ion. In order for a peak to be assigned to a metabolite, it must have a retention index difference of ≤ 10 and a mass spectral similarity of ≥ 70%. Our reasons for choosing two separate criteria for peak identification can be explained using glucose and galactose as examples. Both metabolites have identical mass spectra, but elute at different retention times and will therefore have different retention indexes. Another example is with aspartate and pyroglutamate. Both metabolites elute at the same time, but have very different mass spectra. By using retention indexes and mass spectral similarity, we can deconvolute and positively identify all 4 of these metabolites. This would not be possible using only one of the criteria.

Once a peak has been matched to a metabolite in our library, its abundance is recorded based on the intensity of the qualifier ion listed in the library for that metabolite. If a peak cannot be assigned to a metabolite, the peak will be annotated as "Time_Mass", where "Time" is the peak retention time and "Mass" is the most abundant ion in the peak's mass spectrum. The unknown peak abundance is recorded based on the highest intensity ion in the mass spectrum.

B5.4 Targeted biomarker analysis of Tissues and Environmental Matrices

For targeted biomarker analysis (e.g. pesticide residues in amphibians and bee matrices), the Biomarkers Research Team should follow the target QC criteria listed below:

Typical sample sets should include: method blank (procedural blank), matrix blank (control), and a matrix spike at 2x LOQ with all analytes. Matrix blanks can be used to prepare matrix matched standards. When a matrix blank is not available, samples may be analyzed to determine those free from the target analytes, which may be then used as matrix blanks. If no samples are suitable for matrix blank, a blank from another related matrix, known to be free from the target analytes, may be extracted and run with its set of matrix standards at the same time samples with no matrix blank are analyzed to monitor extraction efficiency.

The method (procedural) blank should be free of contaminants at the limits of detection for all the target analytes. If target analytes or interferences are detected at these levels, causes of the contamination should be investigated, and the samples may need to be re- extracted and re-analyzed or the limit of detection increased to an appropriate level.

An instrument calibration curve, with a minimum of five concentration levels, (typically between estimated LOD-1Ox LOQ) will be established for each analyte prior to the analysis of actual samples. The correlation coefficient (r^2) of the calibration curve, for each quantified analyte, should be >0.97. Samples will be quantified within the demonstrated instrument calibration range; however, quantitation within± 10% of the demonstrated calibration range is permitted.

At least one calibration check standard (generally a mid-level calibration standard solution) at a minimum rate of not less than 5% (1 in 20 injections) will be analyzed within an analytical sequence. The relative percentage difference (RPD) or% deviation between the calibration check standard and the initial calibrations should be within 20% for all the analytes.

Control matrix blanks, spikes and method (procedural) blanks should be used to determine recovery, bias and precision. The recovery of spiked compounds from the spiked matrix blank should ideally be within the range of 70% to 120% at levels equal to, or above, 2x LOQ, for the parent imidacloprid. However, due to the more polar nature of some metabolites, recoveries below 70% are expected according to the method validation results.

The identity of analytes should be determined and confirmed by retention time (±0.1 min.) and by monitoring two precursor-product ion transitions in the LC-MS/MS process, which agree within± 20% of the relative ion ratios of the standards.

B6. Instrument/Equipment Testing, Inspection, and Maintenance

Cleaning and preventative maintenance of small laboratory equipment, such as changing of pipettors o-rings and cleaning of balances, is done during yearly calibration services for the pipettors and balances.

All freezers and refrigerators used to store samples or reagents are checked and recorded weekly. The temperatures of incubators will be checked and recorded daily when in use. If temperatures exceed acceptable limits, researchers are notified and corrective action (such as temporarily relocating samples until issue is resolved) is taken and documented in research notebooks.

The analysts maintain the laboratory analytical equipment on a continuous basis. The sensitivity of the mass spectrometers are carefully monitored using quality control/calibration standards, and when performance deteriorates, action will be taken to remedy the problem. Logbooks will be maintained for each instrument. Maintenance will be provided as described in the instrument operation manuals. The instrument logbooks will be kept current and complete, and will contain the lists of samples analyzed, data file names, conditions used, and maintenance records. In addition, analytical run and instrument conditions can be accessed electronically for each file.

Before a series of analyses is conducted on these systems, they must be tuned and their sensitivity assessed with respect to check standards. Overall acceptability of instrument tune performance will be determined according to the manufacturers' specifications.

B7. Instrument/Equipment Calibration

B7.1 Laboratory Equipment

Calibration of lab equipment (micropipettes and balances) are performed annually by an external ISO 17025 accredited vendor under a contract managed by NERL's QA office. Calibration stickers are placed on each piece of equipment and calibration certificates are maintained in the NERL-Athens QA office. Equipment should be first be inspected to ensure that it has not surpassed the calibration due date prior to use and equipment that has exceeded this date should either be removed from service or verified that it is performing properly. This verification should be documented in the research notebook or logbook.

Balance calibration checks are performed daily with use by working weights which are annually verified by the Athens QA office and traceable to NIST. Results of balance checks are documented in the balance log book. Failure to meet this test requires recalibration of the balance. Continued failure at this point requires maintenance by a certified service technician.

B7.2 Major Analytical Instrumentation

The Biomarkers Research Team maintains the laboratory analytical equipment on a continuous basis. The accuracy, precision, and sensitivity of each instrument are carefully monitored prior to analysis of each dataset using multiple analyses of samples containing known concentrations of analytes (see Section B5). When any of these parameters deteriorates by >10%, action will be taken to remedy the problem. Logbooks will be maintained for each instrument. Maintenance will be provided as described in the instrument operation manuals. Overall, all the analytical equipment used in this study will be calibrated prior to sample analysis and quality assurance standards are incorporated into routine use to verify instrument response both before and during sample runs.

B8. Inspection/Acceptance of Supplies and Consumables

All analytical standards, reagents, and solvents will be of the highest purity available, and must not contain contaminants that would interfere with the detection of analytes. Potential contamination of the standards, reagents, and solvents will be determined during a quality control run. Inspection of supplies and consumables will be performed by each investigator.

B9. Non-direct Measurements

Not directly applicable to this project.

B10. Data Management

Researchers are required to adequately document in research notebooks all aspects of research as outlined in ORD PPM Section 13.2 Scientific Recordkeeping: Paper. Researchers may adopt some of the guidance outlined in ORD PPM Section 13.6 Scientific Recordkeeping: Use of Electronic to manage and archive electronic files. All information associated with an experiment (e.g., raw MS spectra, Excel spreadsheets and associated calculations) is stored in folder locations that are referenced in the analyst's research notebook and/or in the Table of Contents section. Biomarker Research Team Members' Notebooks are reviewed and signed by the technical lead on a semiannual basis and by the supervisor (i.e. PI's branch chief) annually.

Data is transferred by hand or electronically to a location where chemical data reduction or conversion will be carried out. The primary location of these electronic records is the PI's office computer. Backups of these records will be made to the PI's "My Documents" drive on the network at least quarterly. Offsite backups of the network drive are maintained by the EPA contractor.

Raw chromatographic data will be acquired automatically by instrument specific software, stored on computer hard drive during the project, and archived on digital media. Copies of chromatograms and other lab records will be maintained in the project file. Data will be processed using the analytical instrument software, or will be transferred into a spreadsheet program for data manipulation.

The researcher(s) are responsible for data management while the project is ongoing, the researchers will archive the files following Agency guidelines in accordance with applicable EPA Records Schedules (currently EPA Records Schedule 1035 for Environmental Programs and Projects). The logic, accuracy and transcription of data transferred or of calculated results should be checked, initialed and dated by a second researcher or co-PI, other than the researcher performing the calculations. Data may be reviewed electronically and may be digitally signed to reduce paper consumption.

Additionally, data stored in digital format will be backed-up over the network (L: drive) when necessary for cross-ORD projects, a request will be submitted for space on the O: drive and access granted to project-specific individuals. The same formatting and file

tree will be implemented as for local projects. A record of data backups is maintained in instrument specific log books.

Table 1: General summary of data management and file trees for data storage.

Type of Data	Storage medium	Storage location
Experimental Project Data (NERL research)	L drive Public Folder	L:\Public\Henderson\Project Title\User
Experimental Project Data (Cross-ORD Research)	O Drive Public Folder	O:\Public\Project Title\Henderson\User
Laboratory procedures and notes	Laboratory notebook	Bound, prenumbered NERL issued research notebook(s) kept in the analysts' office for the duration of the project, or electronic OneNote notebook on SharePoint
GC/MS and LC/MS instrument checks (e.g. tune files, sequence data, and user runs)	Log books	With each instrument
Small instrument checks (e.g., balances, temperature monitoring of storage units)	Log books	With each instrument

C. Assessment and Oversight

C1. Assessment and Response Actions

C1.1 Routine Technical Oversight

The technical lead will have responsibility for monitoring day to day project activities and identifying or confirming quality problems. Any problems identified by scientific support staff will be brought to the attention of the technical lead who will document the nature of the problem, initiate corrective actions, and ensure the recommended corrective action is carried out. The technical lead will perform surveillance activities to ensure that management and technical aspects are implemented properly as defined in this QAPP. These activities will include assessing how project milestones are achieved and documented, corrective actions are implemented, reviews are performed, and data are managed.

Many technical problems that might occur such as modifying the technical approach or correcting errors or deficiencies in documentation can be solved immediately by the technical staff. The project team is responsible for documenting a response to any

significant findings. Immediate corrective actions are part of normal operating procedures and noted in project records such as research notebooks. Problems that cannot be solved in this way may require more formalized corrective action. If quality problems that require such attention are identified, the technical lead will contact the QA Manager to determine whether attaining acceptable quality requires short- or long-term actions.

C1.2 Quality Assurance Audits

The designated QA Manager for this project may conduct a Technical Systems Audit (TSA) during execution of the research project to ensure this QAPP is being implemented as intended. The objective of the TSA is to evaluate the adequacy of facilities, measurement equipment, recordkeeping, sample handling/storage, operating procedures, reporting requirements and QC procedures.

Additionally, the QAM, or a designee, may perform an audit of data quality (ADQ) on either all or a portion of the generated data. ADQs are examinations of data after collection and verification by project personnel. It is conducted to determine how well the measurement system performed with respect to the performance goals specified in the QAPP, SOP or other QA document and to provide a detailed review of: (1) the electronic or manual recording and transfer of raw data; (2) data calculations; (3) documentation procedures; (4) the selection and discussion of appropriate data quality indicators (i.e., precision, accuracy, completeness, comparability and representativeness); (5) security procedures; and (6) back-ups.

Corrective actions will be required to address any findings identified during QA audits. The technical lead is responsible for coordinating the implementation of corrective actions and submitting any requested documentation to the QAM as evidence of their implementation. The QAM may also choose to conduct follow up activities to assess the effectiveness of corrective actions, as deemed necessary.

C1.3 Review of Scientific and Technical Products

Technical products produced in this study that present environmental data or include models or existing data are required to undergo both internal technical review and QA review prior to release as required by the ORD Clearance Policy. The results of each review will be documented in the clearance package. QA review will be documented on NERL's Scientific and Technical Product QA Review Form or equivalent. Technical EPA 600-series reports will include a readily identifiable QA section that will include a summary of the QA activities performed during this project.

The QAM can provide guidance on information to be documented in this section, if necessary.

C2. Reports and Management

No fixed schedule for reporting research progress exists, but managers (Branch Chiefs and the Division Director) are kept informed by oral and written communication. All research is, however, periodically reviewed and this involves either written or oral presentations. Primarily research progress is reported through research products (publications, modeling tools, etc.). Results that are to be published in peer-reviewed literature, will be subjected to both internal (EPA) peer-review as well as the external peer-review process required by the journal editor. All Division managers in the chain-of-command will review and clear these products.

As necessary, the Branch Chief should be informed of the progress and development of the project on a weekly basis and documented. As necessary, interim reports of major milestones may be issued, consisting of a summary of current progress, associated QC procedures, and conclusions at that step. Final, authoritative datasets and associated metadata will be reported upon the completion of the analyses as specified in the scientific data management plan. The final report should include sample residue results, and associated QC sample data.

D. Data Validation and Usability

D1. Data Review, Verification, and Validation

On a quarterly basis, the PI will verify that the quality objectives and criteria (section A7) are met throughout the course of the project and that proper documentation as stated in section A9 is followed. This review will be documented in the PIs laboratory notebook and the current QAPP amended as necessary. All articles and presentations go through the NERL clearance process. In addition, laboratory procedures, notebooks, and electronic data will be subject to a Technical Systems Audit (TSA) to be performed by the EMMD QA Manager. The data generated in this QAPP will undergo QA review by a secondary reviewer (e.g. co-PI) to check for completeness, calculations, and transcription errors before preparing the data for publication or any form of presentation. The data will be reported with appropriate data quality flags, as needed and documented in the electronic file (i.e. Excel spreadsheet).

Data Reduction and analysis can be performed using instrument-specific software or by importing the data into commercially available spreadsheet or statistical software for user manipulation. The instrument software report results in peak area

counts and is exported as excel spreadsheets. The ng/L or ng/g assignments are done in the instrumentation software based on the user assignments for the calibration curve and type of sample. The excel spreadsheets will contain all analytical data required to meet the QA objectives, including target analyte concentrations expressed as ng/L, as appropriate, limits of quantitation (LOQ) or limits of detection (LOD), comments, and methodological blanks and concurrent QC sample results. All analytical data will be linked to sample ID number and also associated with time and date of sample analysis.

D2. Verification and Validation Methods

Most, if not all, outputs from this project will be peer reviewed journal articles. Success can be judged through acceptance of articles in respected journals. Else, subject to the judgement of the PI and Co-PIs, further verification procedures such as performance-evaluation studies or blind samples may be undertaken. Virtually all outputs from this task will be peer reviewed in submission for publication as journal articles, thus providing another level of verification and validation. Additional internal review of work products will be performed as deemed necessary by branch, division or laboratory management.

Data verification will be conducted to ensure that complete and accurate analytical information is available for all samples analyzed by EMMD laboratories. Data verification begins during and after the period of analysis, and data entry into the Excel spreadsheet. The key personnel of the analytical team will perform the first level of review, ensuring that all data have been validated. The mechanisms used for all data transcriptions and transmissions will be reviewed, and a random subset of all transcriptions checked. For data requiring calculation of results, a random subset (approx. 5%) of the raw data will be recalculated.

Once the chemical measurement data have been exported into Excel, the following QA/QC checks to validate the data will be carried out:

- Sample ID checks to verify that all Sample IDs with reported data are valid Sample IDs, i.e., they were logged in as received from federal or non-federal laboratories

- Missing data checks to verify that all Sample IDs received from the field either had a full set of analytical data reported or were disqualified, as documented in the CoC data,

- Duplicate data checks, to verify that the same analytical data were not imported into the Excel spreadsheet twice for a given sample,

- Out-of-range checks, to verify that all data for data fields limited to a code set did not violate that code set,

- Calculation verification; for any calculations performed within the Excel spreadsheet a random subset of the raw data are calculated using an independent calculation source (Excel spreadsheet) for validation.

- The above QA/QC checks will be documented on a readme tab in electronic spreadsheets when available or in an addendum for other electronic files containing but not limited to the dates reviewed, the reviewer, results of the review and any corrective actions taken.

D3. Reconciliation with User Requirements

Data quality flags will be assigned to each chemical measurement record as needed to identify the quality and usability of the record. Data quality flags will be assigned as documented in each report, if necessary. These results will be provided to the NHEERL and other EPA collaborators and to clients in the Program Offices and Regions. During all aspects of the research (planning, execution, reporting) we confer frequently with collaborators and clients to ensure that validation requirements of products are met and fulfill their needs.

E. References

Niu, W., Knight, E., Xia, Q., & McGarvey, B. D. (2014). Comparative evaluation of eight software programs for alignment of gas chromatography–mass spectrometry chromatograms in metabolomics experiments. *Journal of Chromatography A*, *1374*, 199-206.

Collette, T. W., Teng, Q., Jensen, K. M., Kahl, M. D., Makynen, E. A., Durhan, E. J., ... & Ekman, D. R. (2010). Impacts of an anti-androgen and an androgen/anti-androgen mixture on the metabolite profile of male fathead minnow urine. *Environmental science & technology*, *44*(17), 6881-6886.

Viant, M. R. (2007). Revealing the metabolome of animal tissues using 1 H nuclear magnetic resonance spectroscopy. In *Metabolomics* (pp. 229-246). Humana Press.